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Short Communication

Inhibitory effect of ascorbic acid on the proliferation and invasion of hepatoma cells in culture

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Abstract

Effect of ascorbic acid (AsA) on the proliferation and invasion of rat ascites hepatoma AH109A cells was investigated by measuring [^{3}H]thymidine incorporation into acid-insoluble fraction of the cells and by co-culturing the hepatoma cells with rat mesentery-derived mesothelial cells, respectively. AsA suppressed the invasion of AH109A cells in a dose-dependent manner at concentrations of 62.5–500 μM, while it inhibited the proliferation of the cells at higher concentrations of 250 and 500 μM. Hepatoma cells previously cultured with hypoxanthine (HX) and xanthine oxidase (XO) or with hydrogen peroxide showed increased invasive activities. AsA suppressed the reactive oxygen species-potentiated invasive capacity by simultaneously treating AH109A cells with AsA, HX and XO or with AsA and hydrogen peroxide. Furthermore, AsA reduced the intracellular peroxide levels in AH109A cells. These results suggest that the antioxidative property of AsA may be involved in its anti-invasive action on hepatoma cells.

Abbreviations: AsA – ascorbic acid; CS – calf serum; HX – hypoxanthine; M-cells – mesothelial cells; MEM – Eagle's minimum essential medium; ROS – reactive oxygen species; XO – xanthine oxidase

Endless proliferation and metastasis are two biological properties of cancer cells. Metastasis is the primary cause of death in human cancer. Cancer metastasis is attained by a sequence of steps (Fidler et al. 1978), of which invasion is particularly complicated process and key step in the metstatic cascade (Liotta et al. 1991; Al-Mehdi et al. 2000). Furthermore, metastasis and invasion may be correlated to reactive oxygen species (ROS) (Nonaka et al. 1993; Kozuki et al. 2000). Some food factors with antioxidative activity inhibit the invasion of hepatoma cells (Zhang et al. 2000; Kozuki et al. 2001a, b). Ascorbic acid (AsA), one of the water-soluble vitamins, is a well-known biologically important antioxidant. This vitamin takes part in many biological processes such as collagen and hormone syntheses, hemostasis, and protection of lipid membranes which might affect chronic disease risk (Jialal et al. 1990; Ness et al. 1996; Khaw et al. 2001). The present study was attempted to define the effect of AsA *in vitro*, focusing our attention on the invasion of hepatoma cells.

Materials and methods

Materials

L-Ascorbic acid (AsA) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. AsA was dissolved in medium, and it was sterilized

with filtration. AsA was of analytical grade and used without further purification.

Culture of AH109A hepatoma cells

A rat ascites hepatoma cell line of AH109A was provided by the Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan. AH109A cells were maintained in the peritoneal cavities of male Donryu rats, prepared from accumulated ascites and cultured *in vitro* in Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% calf serum (CS, obtained from GIBCO BRL, Grand Island, NY) (10% CS/MEM) as described previously (Kozuki et al. 2000). To eliminate contaminated macrophages and neutrophils, these cells were cultured at least for 1 week after isolation and used for the assays described below.

In vitro proliferation assay

Effect of AsA on the proliferation of AH109A cells was examined by measuring the incorporation of [methyl$-^3$H] thymidine (20 Ci/mmol, PerkinElmer Life Science, Inc., Boston, USA) into acid-insoluble fraction of cells for 4 h as described previously (Yagasaki et al. 1992).

In vitro invasion assay

Effect of AsA on the invasion of AH109A cells was examined by the co-culture system (Akedo et al. 1986) with slight modifications as described previously (Kozuki et al. 2000). Briefly, mesothelial cells (M-cells) were isolated from mesentery of male Donryu rats (6–8 weeks old). After digestion by trypsin, 1.2×10^5 M-cells were plated in a 60-mm culture dish with 2-mm grids (Nunc A/S, Roskilde, Denmark), and cultured for 5–7 days to a confluent state in 10% CS/MEM. Then, AH109A cells (2.4×10^5 cells per dish) were applied on the monolayer of M-cells in 10% CS/MEM with AsA for 24 h. Invaded cells and colonies underneath M-cells were counted with a phase-contrast microscope. Usually 10 areas were counted, and the invasive activity of AH109A cells was expressed as the number of invaded cells and colonies/cm^2.

Pretreatment of AH109A cells with hypoxanthine and xanthine oxidase or hydrogen peroxide

AH109A cells were cultured for 4 h in the absence or presence of 62.5 μM AsA with or without a ROS-generating system, i.e., 4 μg/ml hypoxanthine (HX, Sigma, St. Louis, MO) with 7×10^{-4} U/ml xanthine oxidase (XO, Sigma) (Shinkai et al. 1986; Tanaka et al. 1997) or 25 μM hydrogen peroxide (H_2O_2, Wako Pure Chemical Industries). AH109A cells were then washed once with 10% CS/MEM and seeded on the M-cell monolayer in 10% CS/MEM without AsA and ROS. After cultured for 24 h, invaded cells and colonies underneath M-cells were counted with a phase-contrast microscope as described above.

Flowcytometric analyses of intracellular peroxide in AH109A cells

Intracellular peroxide levels in AH109A cells were assessed by flow cytometric analyses using a fluorometric probe (2′,7′-dichlorofluorescin diacetate ; DCFH-DA, Molecular Probes, Eugene, OR) (Bass et al. 1983) with EPICS ELITE EPS (Beckman-Coulter, Hialeah, FL) as described previously (Miura et al. 2003).

Statistical analysis

Data were expressed as means $\pm$SEM. Multiple comparison was performed by one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test, and $p < 0.05$ was considered statistically significant.

Results

Effect of AsA on the proliferation and invasion of AH109A cells

We first investigated the effect of AsA on the proliferation and invasion of AH109A cells (Figure 1). AsA exerted little influence on the AH109A

proliferation at concentrations up to 125 μM and significantly suppressed it at higher concentrations of 250–500 μM (Figure 1a). In contrast, AsA commenced to significantly suppress the AH109A invasion at a low concentration of 62.5 μM, suppressed it approximately linearly to 125 μM, and maintained the inhibitory effect up to 500 μM (Figure 1b). These results suggested that the inhibitory action of AsA might be stronger on the invasion than on the proliferation.

Effect of AsA on the invasion of AH109A cells pre-treated with hypoxanthine and xanthine oxidase or hydrogen peroxide

To examine whether or not AsA would inhibit the invasion of tumor cells by its antioxidative activity, the invasion assay was performed with AH109A cells pre-cultured in ROS-containing medium. As shown in Figure 2a, the invasive activity of AH109A cells pre-cultured in the HX–XO system which generates ROS was significantly higher than that of AH109A cells with no treatment. AsA inhibited the ROS–potentiated invasive activity of the pre-cultured cells when added to the medium at a concentration of 62.5 μM on pre-culturing the hepatoma cells with HX and XO. Likewise, the invasive activity of AH109A pre-treated with hydrogen peroxide was significantly elevated, and this rise was canceled by adding AsA (62.5 μM) to the medium containing hydrogen peroxide (Figure 2b). AsA exerted no influence at 62.5 μM on the AH109A proliferation (Figure 1a). Thus, the concentration of 62.5 μM was adopted in these experiments to avoid influences of the AsA-mediated proliferation inhibition on the AH109A invasion.

Intracellular peroxide levels of AH109A cells pre-treated with hypoxanthine and xanthine oxidase or hydrogen peroxide

AH109A cells treated by HX–XO (Figure 3a) or hydrogen peroxide (H_2O_2, Figure 3b) for 1 h contained more intracellular peroxides than did control cells (control vs. HX–XO, control vs. H_2O_2) when analyzed with a flow cytometer using DCFH-DA as an indicator. AsA (62.5 μM)

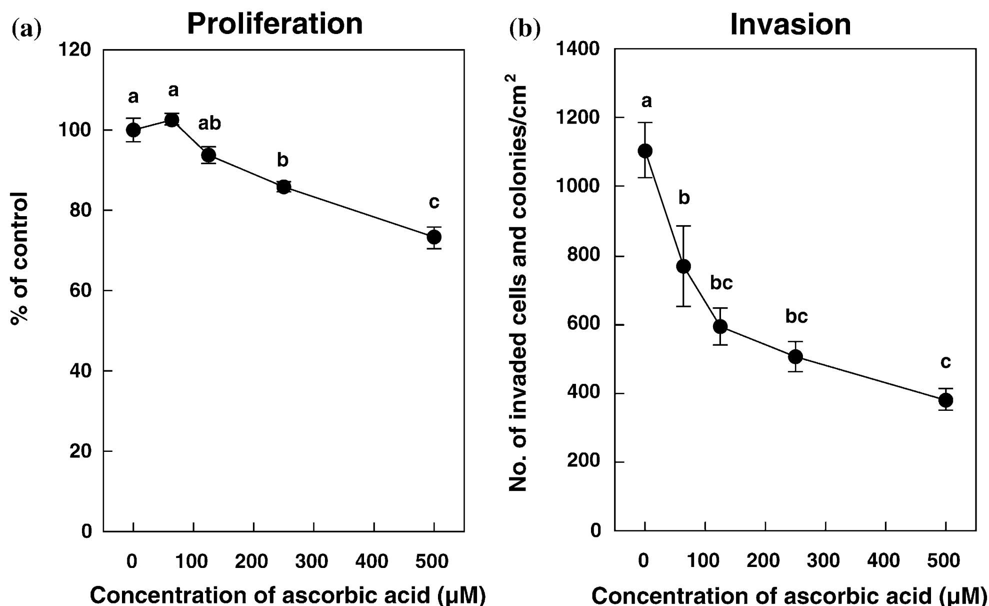

Figure 1. Effect of AsA on the proliferation (a) and invasion (b) of AH109A cells. AsA was directly dissolved in culture medium at the concentrations indicated in figures. The proliferative activity of AH109A cells (a) was determined by the incorporation of [methyl–^{3}H] thymidine and the invasive activity (b) by invasion assay as described in Materials and methods section. Data are the means ± SEM of six wells (a: proliferation) and 10 areas (b: invasion). Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey–Kramer multiple comparisons test.

inhibited these rises in the intracellular peroxide levels of AH109A cells (HX–XO vs. HX–XO + AsA, H_2O_2 vs. H_2O_2 + AsA). AsA was also found to scavenge endogenous intracellular peroxides (control vs. AsA).

Discussion

In this study, we demonstrated that AsA could inhibit both the proliferation and invasion of AH109A cells *in vitro* (Figure 1). AsA concentrations which could suppress the proliferation of AH109A cells (Figure 1a, 250–500 μM) were higher than those which could suppress the invasion of AH109A cells (Figure 1b, 62.5–125 μM). AsA is reported to inhibit the proliferation of other tumor cells, for instance, BL6 melanoma *in vitro* at high concentrations up to 1100 μM (Gardiner and Duncan 1989). In case of BL6 melanoma, however, dietary supplemented AsA reduced the growth of BL6 melanomas transplanted in C57 mice (Gardiner and Duncan 1989). Thus, there is a possibility that dietary supplemented AsA may also has potential to suppress the AH109A hepatoma growth *in vivo* despite the requirement of high doses for the suppression *in vitro*. On the contrary, AsA significantly suppressed the invasion of the hepatoma cells at concentrations of 62.5–125 μM which might be the physiological plasma levels of the vitamin (Levine et al. 1996).

Our previous works have demonstrated that the invasion of AH109A cells is accelerated by ROS (Kozuki et al. 2000). In the present study, we therefore examined the effect of AsA on the ROS-potentiated invasive activity using both HX–XO system and H_2O_2. AsA was found to inhibit the ROS-induced elevation of the AH109A invasion (Figure 2). Furthermore, intracellular peroxide levels in AH109A cells were measured with a flow cytometer. AsA was found to scavenge both exogenous and endogenous intracellular peroxides (Figure 3). The latter peroxide may be a cause for spontaneous invasion of AH109A cells, because tumor cells are known to produce a large amount of ROS compared with normal cells (Szatrowski and Nathan 1991). Phosphorylated ascorbate is also reported to inhibit tumor invasion by decreasing oxidative stress (Nagao et al. 2000).

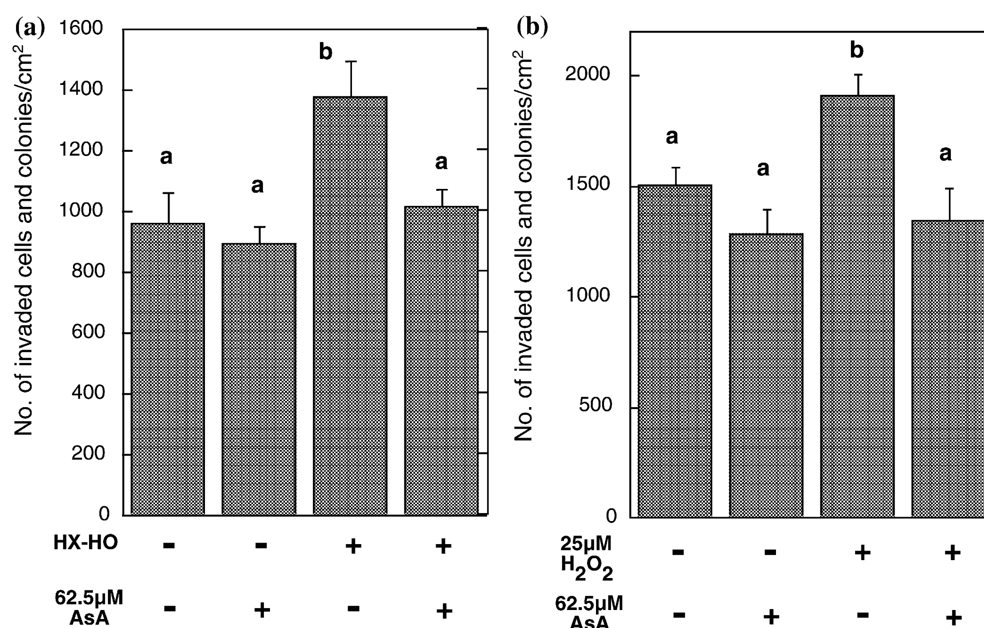

Figure 2. Effect of AsA on the invasion of AH109A cells pre-cultured with HX–XO (a) or hydrogen peroxide (b). AH109A cells were cultured for 4 h in the absence or presence of 62.5 μM of AsA and/or HX (4 μg/ml) with XO (7×10^{-4} U/ml) or 25 μM of hydrogen peroxide (H_2O_2). After the treatment, AH109A cells were washed and applied onto the monolayer of M-cells in medium. The invasive activity was determined as described in Materials and methods section. Data are the means ±SEM of 10 areas. Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey–Kramer multiple comparisons test.

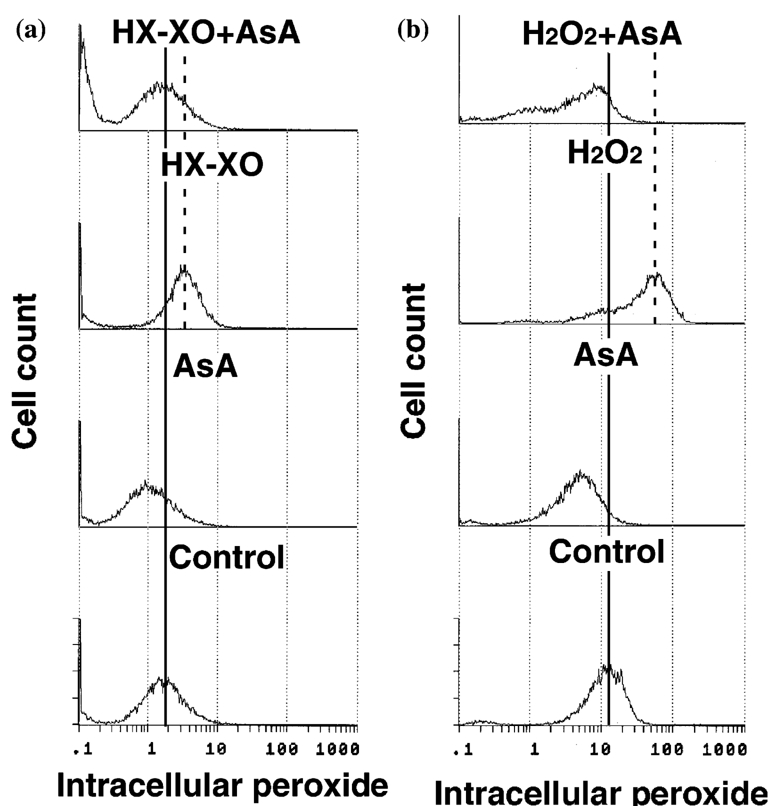

Figure 3. Flowcytometric analyses of intracellular peroxide in AH109A cells treat with exogenous reactive oxygen species (ROS) and AsA. AH109A cells were treated with HX–XO (a) or hydrogen peroxide (H_2O_2) (b) as ROS resources. After ROS treatment for 1 h, DCFH-DA was added at a final concentration of 64 μM and the cells were incubated for 20 min. Basal and ROS activated intracellular peroxide levels were indicated as solid and dotted line, respectively. A representative result is shown.

These findings in previous and present studies strongly suggest that the antioxidative property of AsA may be involved in its anti-invasive action. Potentiation of invasive activity of AH109A cells by ROS is reportedly mediated by autocrine/paracrine loop of hepatocyte growth factor (HGF) (Miura et al. 2003), which is known as a cell motility factor (Parr and Jiang 2001). AsA is likely to suppress the ROS-induced increases in AH109A invasion by interrupting this loop.

In the invasion assay system employed here, AH109A cells are co-cultured with M-cells. We have found that ROS can induce gene expression of HGF in M-cells as well as AH109A cells (Miura et al. 2003). Thus, HGF produced by both AH109A and M-cells may potentiate the motility of AH109A cells and also may induce the retraction of M-cells, leading to acceleration of the AH109A invasion. Provided that AsA, like a polyphenol resveratrol (Miura et al. 2004), suppresses the production of HGF through its antioxidative activity, the vitamin may diminish the induction of the retraction of M-cells as well as the motility of AH109A cells, this leading to the effective reduction of the AH109A invasion by reducing the functions of both cells at the same time. This also may explain, at least partly, why AsA suppresses more strongly the invasion than the proliferation of AH109A cells (Figure 1). Further intensive studies are required to clarify this possibility.

In the absence of exogenous ROS, AsA significantly suppressed the AH109A invasion at 62.5 μM (Figure 1b), while AsA at the same concentration failed to do so (Figure 2). This discrepancy may be due to the difference in the experimental conditions, that is, continuous exposure of cells to AsA during invasion assay for 24 h (Figure 1b) vs. transient exposure of cells to AsA for only 4 h followed by invasion assay for 24 h in the absence of AsA

(Figure 2), suggesting that the effect of AsA on the AH109A invasion may be time-dependent. Under the experimental conditions used in Figure 2, AsA canceled the ROS-induced rise in invasive activity and returned it to the control level. However, AsA may further decrease the invasive activity below the control level even in the presence of exogenous ROS at higher concentration and longer exposure time. Thus, AsA is suggested to suppress the ROS-induced and spontaneous invasion of AH109A cells dose- and time-dependently.

In summary, we clearly demonstrated that AsA inhibited the proliferation and invasion of AH109A cells in culture, the invasion being more strongly suppressed than was the proliferation. AsA suppressed the ROS-induced increases in invasive capacity and intracellular peroxide levels. These results suggest that the antioxidative property of AsA may be involved in its anti-invasive action. AsA may have promising beneficial effects in preventing tumor metastasis and may be of significance from the aspect of nutritional control of cancers.

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